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## (54) GENE MUTANT ANIMALS

(57) A gene-mutated animal such as a mouse which comprises a mutant prsenilin-1 gene comprising a DNA having a sequence encoding a mutant presenilin-1 protein in which an amino acid is substituted with a different amino acid in an amino acid sequence of a presenilin-1 protein; for example, a mutant presenilin protein in which isoleucine at position 213 is substituted with an amino acid other than isoleucine, e.g., threonine, in a mouse presenilin-1 protein. The animal is useful as an animal model which has pathological conditions closer to a human patient with Alzheimer's disease.

Fig. 2

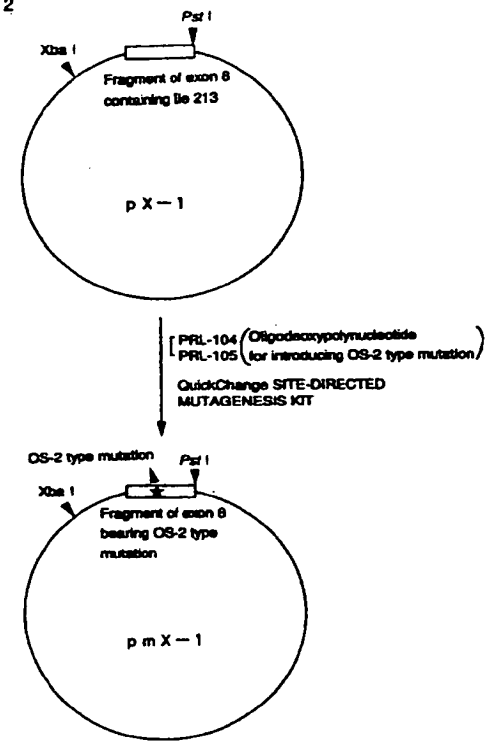


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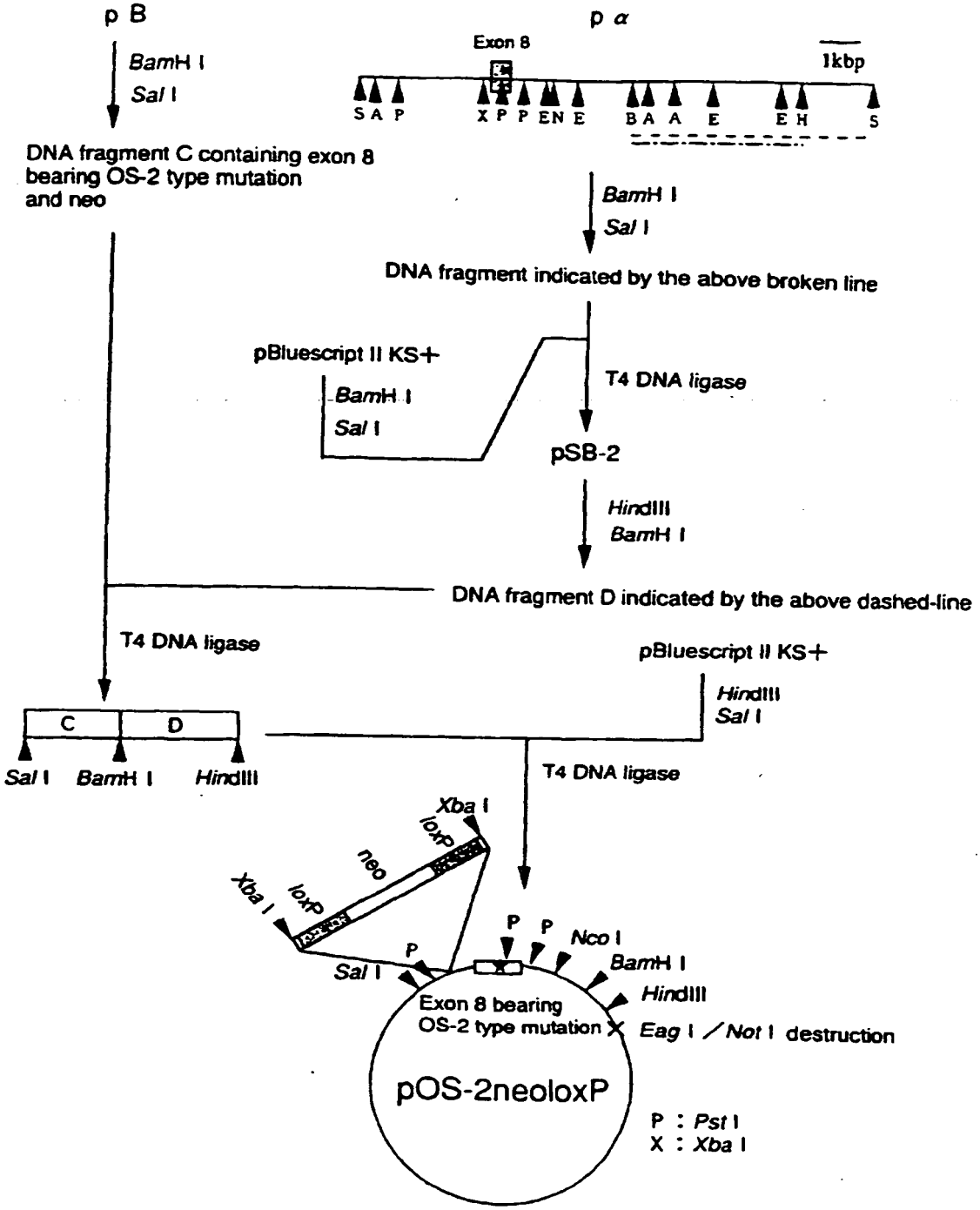
Fig. 7



gested to possibly participate in a certain step of intercellular signal transduction.

**[0010]** The first report on presenilin-1 protein describes that mutations causing the familial Alzheimer's disease are substitutions of amino acid residues at five positions. After this report, genes mutated at various sites were found from many families afflicted with familial Alzheimer's disease, which include OS-2 (isoleucine at position 213 is mutated to threonine) and OS-3 (valine at position 96 is mutated to phenylalanine), both reported by the present inventors (Kamino K. et al., Neurosci., Lett., Vol. 208, P195, 1996), and more than 40 types of amino acid substitutions have been known at more than 30 sites so far (Hardy. TINS, Vol. 20, P154, 1997).

**[0011]** At present, 70-80 % of the familial Alzheimer's disease is believed to be related to the mutation of presenilin-1 protein. Mutations at two sites have been reported as for presenilin-2 protein. As explained above, genetic analysis has proved that mutants of presenilin-1 and presenilin-2 proteins are deeply involved in the familial Alzheimer's disease.

**[0012]** Studies on mechanism how the mutants of presenilin-1 and presenilin-2 proteins cause the onset of Alzheimer's disease have also been progressed. It has been reported that Aβ 40 is almost the same level as normal presenilin-1 and presenilin-2 proteins, whilst Aβ 42 is highly increased as compared to normal presenilin-1 and presenilin-2 proteins in serum or a culture medium of dermal fibroblasts from a patient with Alzheimer's disease having the aforementioned mutants (Scheuner D. et al.: Nature Med., Vol. 2, P864, 1996); in a culture medium of a cell line transformed by mutants of presenilin-1 protein and presenilin-2 protein (Xia W et al.: J. Biol. Chem. Vol. 272, P7977, 1997; Borchelt D.R. et al.: Neuron, Vol. 17, P1005, 1996; Citron, M. et al. Nature Med., Vol. 3, P67, 1997); and in the brain tissue of a patient with familial Alzheimer's disease having the mutant presenilin-1 protein (Lemere C.A. et al.: Nature Med., Vol. 2., P1146, 1996).

**[0013]** These reports show that the mutants of presenilin-1 protein and presenilin-2 protein, which cause the familial Alzheimer's disease, possibly trigger the onset of Alzheimer's disease by the increase of Aβ 42 which is considered to play a significant role in the formation of senile plaque. A trans-genic mouse transferred with a gene encoding the mutant presenilin-1 protein was created (Duff K. et al.: Nature, Vol. 383, P710, 1996, Borchelt DR. et al.: Neuron, Vol. 17, P1005, 1996 and Citron M. et al.: Nature Med., Vol. 3, P67, 1997). It was reported that Aβ 42 in the brain of the trans-genic mouse selectively increased. These results are strong supports of the possibility that mutants of presenilin-1 protein and presenilin-2 protein causing the familial Alzheimer's disease increase Aβ 42 which possibly has significant roles in the formation of senile plaque, thereby develop Alzheimer's disease. However, no description is given about histological study of the mouse's brain in the above reports on the trans-genic mouse, which presumably due to no observation of remarkable histological change in the brain of the trans-genic mouse.

**[0014]** Generally, trans-genic animals are useful as a means of analyzing functions of a target gene *in vivo*. However, it is technically difficult to control the expression of a transferred gene quantitatively, tissue specifically, or time specifically during development. There is also a problem in that two different gene products are present as a mixture in the trans-genic animals since a gene inherently possessed by the animal still works for normal expression, and functions of a transferred gene cannot be sufficiently analyzed. Furthermore, when the transferred gene is subjected to particularly excessive expression, functions not inherently performed in vivo may appear in trans-genic animals, which results in a defect of possible confusion in analysis of constructed gene-mutated animals.

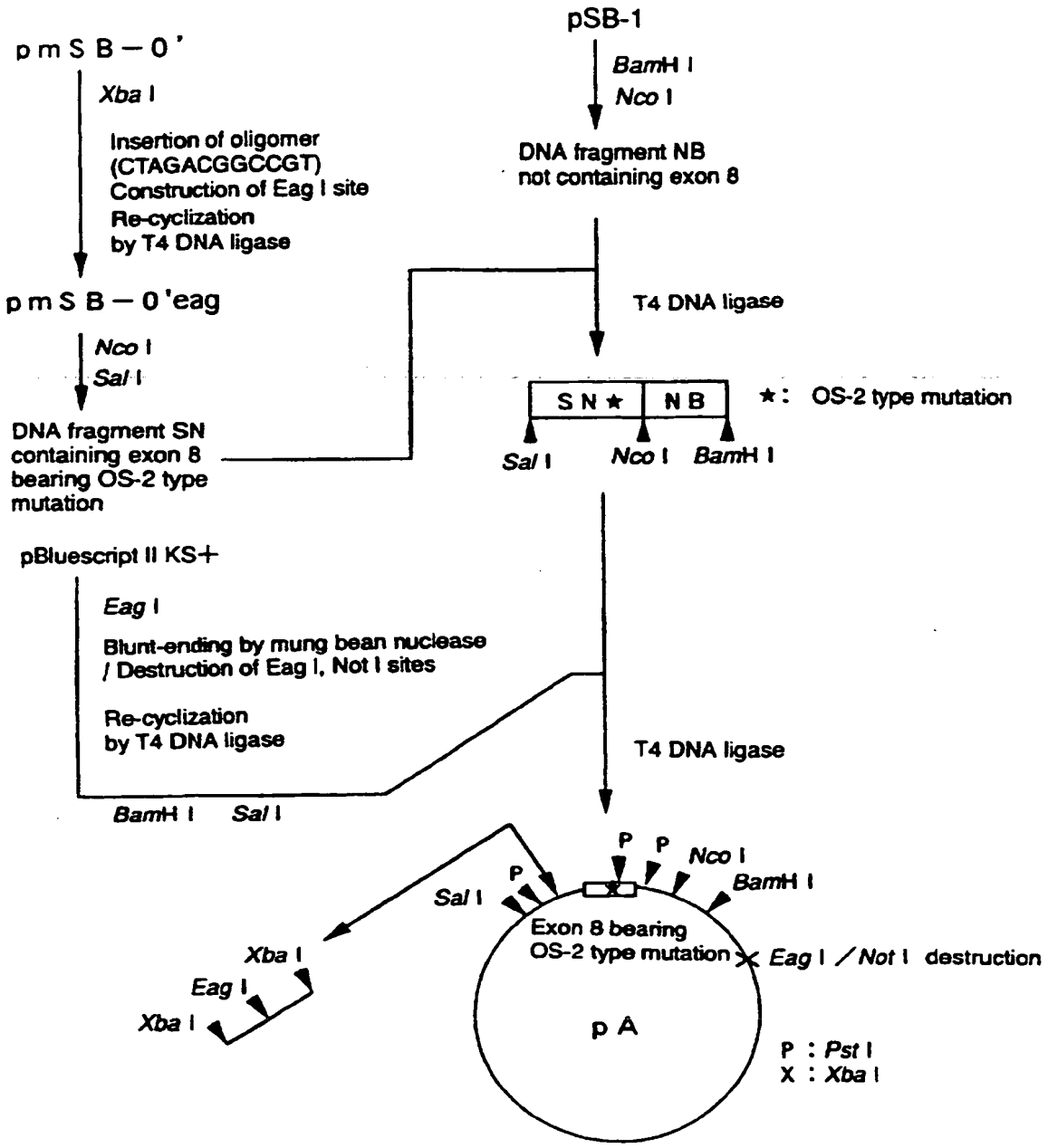
**[0015]** Apart from trans-genic animals, knockout animals may also be used as a means of analyzing functions of a target gene. In a knockout animal, a target gene inherently possessed by the animal is artificially destroyed so as to be dysfunctional. A detailed analysis of knockout animals may reveal functions of a target gene in vivo. However, particular changes in knockout animals created as homozygote sometimes fails to appear, since the functions of the other gene products in the knockout animal may substitute for that of the destroyed gene products. Furthermore, there is also a problem in that an animal as homozygote may sometimes be lethal because the destroyed gene product is essential to the animal's development and growth, whilst thorough analysis of gene functions of an animal as viable heterozygote is practically impossible.

## Disclosure of the Invention

**[0016]** An object of the present invention is to provide, for creation of an animal pathologic model of Alzheimer's disease, an animal as a pathological model whose pathologic conditions is closer to those of a patient with Alzheimer's disease, instead of a trans-genic animal having the aforementioned defects. More specifically, the object of the present invention is to provide a gene-mutated animal capable of expressing a mutant presenilin protein in the brain by transfer of a mutant of a presenilin gene which is believed to be a causal gene of Alzheimer's disease (a mutant presenilin gene) according to a homologous recombination technique. Further objects of the present invention are to provide a method of producing said gene-mutated animal; a plasmid useful for the aforementioned production method; and a method for evaluating a substance or an agent effective for preventive and/or therapeutic treatment of Alzheimer's disease using the aforementioned gene-mutated animal.

**[0017]** In order to reveal roles of presenilin-1 protein and mechanism of the onset of Alzheimer's disease by the mutation of presenilin-1 gene, the inventors of the present invention created a knockin mouse in which presenilin-1

Fig. 5



a mutation of N141I and/or M239V in an amino acid sequence of a presenilin-2 protein.

**[0023]** As preferred embodiments of the aforementioned gene-mutated animals, the present invention provides the aforementioned gene-mutated animal wherein overexpression of amyloid β protein is caused by the mutant presenilin-1 gene and/or the mutant presenilin-2 gene; the aforementioned gene-mutated animal which can express the mutant presenilin protein and wherein the expression of said protein induces the production of amyloid β protein in an amount sufficient to form a progressive neural disease in a peripheral portion of the cerebral cortex of the brain of the animal; the aforementioned gene-mutated animal wherein the animal is a rodent, preferably a mouse: the aforementioned gene-mutated animal wherein the aforementioned mutant presenilin-1 gene and/or the aforementioned mutant presenilin-2 gene are transferred by homologous recombination; the aforementioned gene-mutated animal wherein amount of the amyloid protein expression in a brain tissue induced by the aforementioned presenilin-1 gene is sufficient to cause affected behavior in a memory learning test in comparison with a normal animal, and to induce abnormal neuropathy in a peripheral portion of the cerebral cortex of the hippocampus of the brain of the animal; and the non-human gene-mutated animal having a DNA which comprises a mutant preceilin-1 gene encoding a mutant preceilin-1 protein in which one or two or more amino acids is substituted with a different amino acid in the amino acid sequence of the presenilin-1 protein together with a DNA having a nucleotide sequence encoding a marker protein.

**[0024]** From further aspect, the present invention provides a plasmid comprising a DNA having a sequence of a mutant presenilin-1 gene wherein a DNA sequence encoding around an amino acid at position 213 of a presenilin-1 protein is the following sequence:

5'-TGTGGTCGGGATGATMGCC ANC CACTGGAAAGGCCC-3'

wherein N represents A, G, or C, M represents T or C, and the underlined bases encode an amino acid at position 213; and

a plasmid comprising a DNA having a sequence of a mutant presenilin-1 gene which encodes a mutant presenilin-1 protein wherein an amino acid at position 213 is substituted with an amino acid other than isoleucine in an amino acid sequence of the presenilin-1 protein and a DNA sequence encoding around the amino acid at position 213 of presenilin-1 protein is the following sequence:

5'-TGTGGTCGGGATGATMGCC XYZ CACTGGAAAGGCCC-3'

wherein M represents T or C, XYZ denotes a codon as triplet bases encoding an amino acid other than isoleucine, and the underlined bases encode the amino acid at position 213. Additionally, the present invention also provides a chromosomal DNA containing exon 8 of a mutant presenilin-1 gene encoding a mutant presenilin-1 protein wherein an amino acid at position 213 is substituted with an amino acid other than isoleucine in an amino acid sequence of a presenilin-1 protein.

**[0025]** Furthermore, the present invention provides a plasmid comprising a DNA wherein a Sau3AI site is introduced into a nucleotide sequence comprising the whole or a mutated part of a cDNA or chromosomal DNA of a mutant presenilin-1 gene encoding a mutant presenilin-1 protein in which an amino acid at position 213 is substituted with an amino acid other than isoleucine in an amino acid sequence of presenilin-1 protein. Also provided are the aforementioned plasmid wherein the substitution of the amino acid is isoleucine at position 213 with threonine; and a plasmid comprising a DNA specified by the following nucleotide sequence:

5'-TGTGGTCGGGATGATMGCCACCCACTGGAAAGGCCC-3'

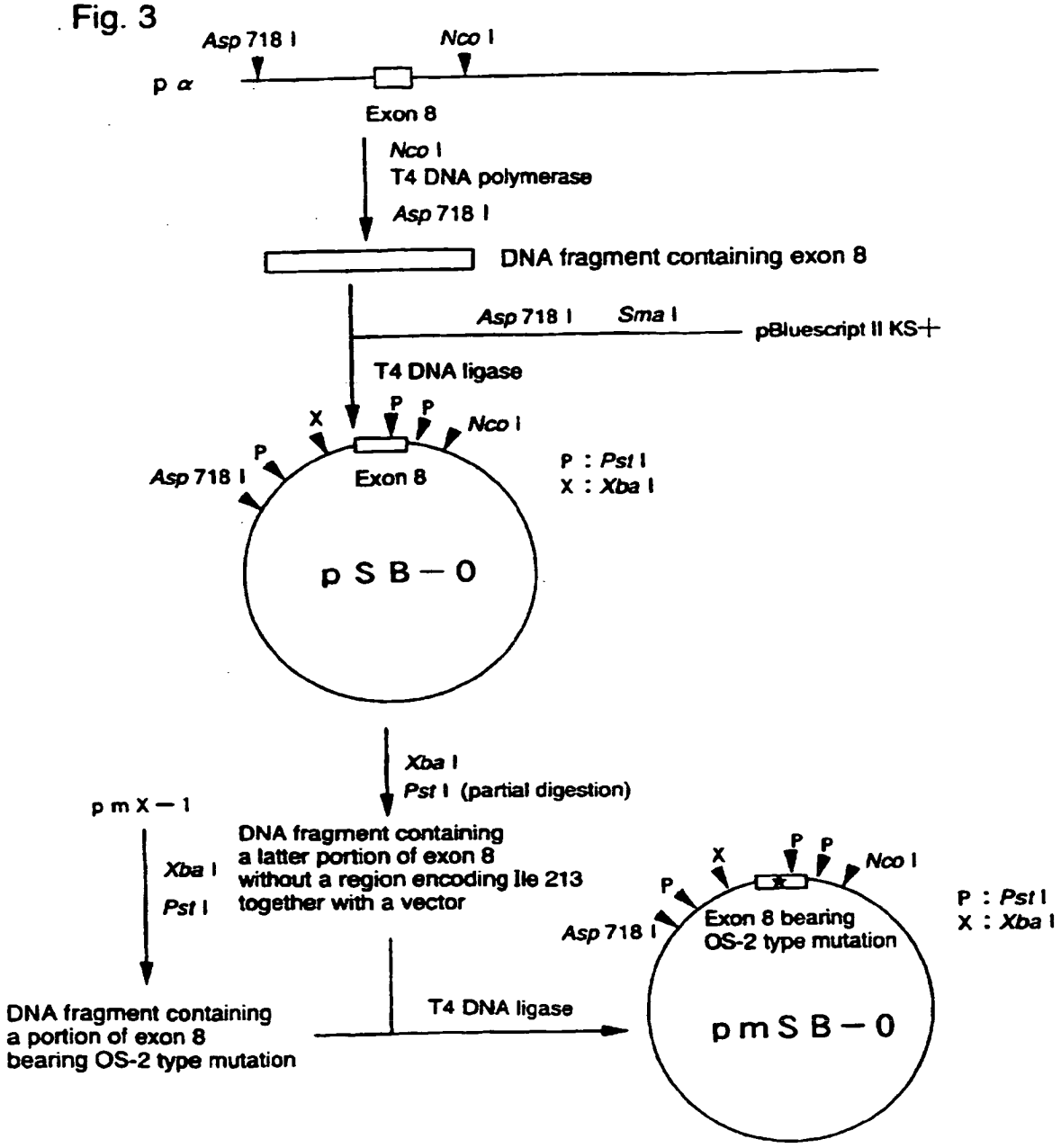
wherein M represents T or C.

**[0026]** In addition to the above inventions, the present invention also provides a gene encoding a mouse mutant presenilin-1 protein wherein isoleucine at position 213 is substituted with an amino acid other than isoleucine in an amino acid sequence of a mouse presenilin-1 protein; and the aforementioned gene wherein the substitution is from isoleucine to threonine. Also provided are a plasmid comprising: (1) a gene encoding a mouse mutant presenilin-1 protein wherein isoleucine at position 213 is substituted with an amino acid other than isoleucine in an amino acid sequence of a mouse presenilin-1 protein; and (2) a neomycine expression unit flanked by loxPs; and the aforementioned plasmid wherein the substitution is from isoleucine to threonine (loxP has been disclosed in Japanese Patent Laid-Open Publication (Kohyo) No. 4-501501, page4).

**[0027]** From further aspect, the present invention provides an embryo introduced with a plasmid comprising a DNA represented by the nucleotide sequence:

5'-TGTGGTCGGGATGATMGCCACCCACTGGAAAGGCCC-3' wherein M represents T or C; an embryo obtained by homologous recombination using each of the aforementioned plasmids; and the aforementioned embryo derived from a mammalian rodent, more preferably from a mouse. The invention also provides a primary cell culture or subcultured cell obtained by isolating a cell from the aforementioned gene-mutated animal and culturing the cell by tissue culture; a method for producing a non-human gene-mutated animal wherein the method comprises the step of transferring a mutant presenilin-1 gene by homologous recombination into an embryo of an animal, wherein the mutant presenilin-1 gene is capable of expressing the mutant presenilin-1 and inducing production of amyloid β protein in an amount sufficient to form a progressive neural disease in a peripheral portion of the cerebral cortex of the brain; and the aforementioned production method wherein a mutant presenilin-1 protein can be expressed wherein isoleucine at position 213 is

Fig. 3



mutant presenilin-1 protein or a mutant presenilin-2 protein. The mutant presenilin gene has the property of increasing the production of amyloid β protein. The gene-mutated animal of the present invention is a mammal transferred with the above-mentioned mutant presenilin gene for example by homologous recombination. The mutation existing in the mutant protein is preferably a result of substitution of an amino acid residue. The number of mutations is not limited, and may preferably be 1.

**[0033]** The full length sequence of a mammal-derived preselin-1 protein is described in, for example, E. Levy-Lahad, et al., Science, 269, pp.973-977, 1995. The full-length sequences of human and mouse presenilin-1 proteins and examples of DNA sequences that encode the proteins are shown in the sequence listings as SEQ ID NOS: 1 to 4. For example, in the mouse-derived presenilin-1, mutation sites may preferably be one or more sites selected from No. 79, No. 82, No. 96, No. 115, No. 120, No. 135, No. 139, No. 143, No. 146, No. 163, No. 209, No. 213, No. 231, No. 235, No. 246, No. 250, No. 260, No. 263, No. 264, No. 267, No. 269, No. 280, No. 285, No. 286, No. 290, No. 318, No. 384, No. 392, No. 410, No. 426, and No. 436.

**[0034]** More preferable mutations are one or more mutations selected from the group consisting of A79V, V82L, V96F, Y115H, Y115C, E120K, E120D, N135D, M139V, M139T, M139I, I143F, I143T, M146L, M146V, H163Y, H163R, G209V, I213T, A231T, A231V, L235P, A246E, L250S, A260V, C263R, P264L, P267S, R269G, R269G, R269H, E280A, E280G, A285V, L286V, S290C, E318G, G384A, L392V, C410Y, A426P, and P436S in the amino acid sequence of the presenilin-1 protein, more preferably in the amino acid sequence of the mouse-derived presenilin-1 protein. Among these mutations, the mutation wherein the amino acid at position 213 is substituted with another amino acid (referred to in some cases as "OS-2 type mutation" in the specification) is a particularly preferable mutation. For example, a mutation wherein isoleucine at position 213 is substituted with an amino acid other than isoleucine, or a mutation wherein isoleucine at position 213 is substituted with threonine is most preferable.

**[0035]** The full-length sequence of a mammal-derived preseline-2 protein is described in, for example, Science, 269, pp. 973-977, 1995. Position 141 and/or position 436 are preferable mutation sites, and in the mouse-derived sequence N141I and/or M239V are more preferable. One or more mutations may exist in either of presenilin-1 protein or presenilin-2 protein, or both of the proteins.

**[0036]** The gene-mutated animal of the present invention is characterized by having the above mutant presenilin-1 gene and/or mutant presenilin-2 gene on its chromosomal DNA. The gene-mutated animal is not limited so far that the animal is a mammal and a kind of the animal is not particularly limited. For example, a rodent may suitably be used. A mouse is particularly preferred. The gene-mutated animal of the present invention can be produced by constructing a plasmid using a DNA having a sequence of about 10kbp comprising a mutant presenilin gene, and then transferring the plasmid into an embryonic stem cell and thereby causing homologous recombination intracellularly.

**[0037]** The gene-mutated animal of the present invention is characterized in that the amino acid mutation occurs mostly at only one position due to the transfer of the aforementioned mutant presenilin-1 and/or presenilin-2 gene by homologous recombination. In the case of a so-called "trans-genic animal", a DNA sequence comprising a mutant portion is inserted randomly into chromosomal DNA, and tens of copies of a repeated sequence are inserted at plural sites. The gene-mutated animal of the present invention can avoid the problems, and it is possible to accurately analyze pathology of Alzheimer's disease at genetic level. Where a DNA comprises a marker or the like is transferred to the gene-mutated animal of the present invention, the animal may have a site of the marker and a sequence for insertion of the market For example, for insertion at a site capable of being cleaved with Sau3AI, one nucleotide can be substituted, and such substitution can be verified by cleaving a PCR product with Sau3AI, followed by subjecting the fragments to electrophoresis or the like.

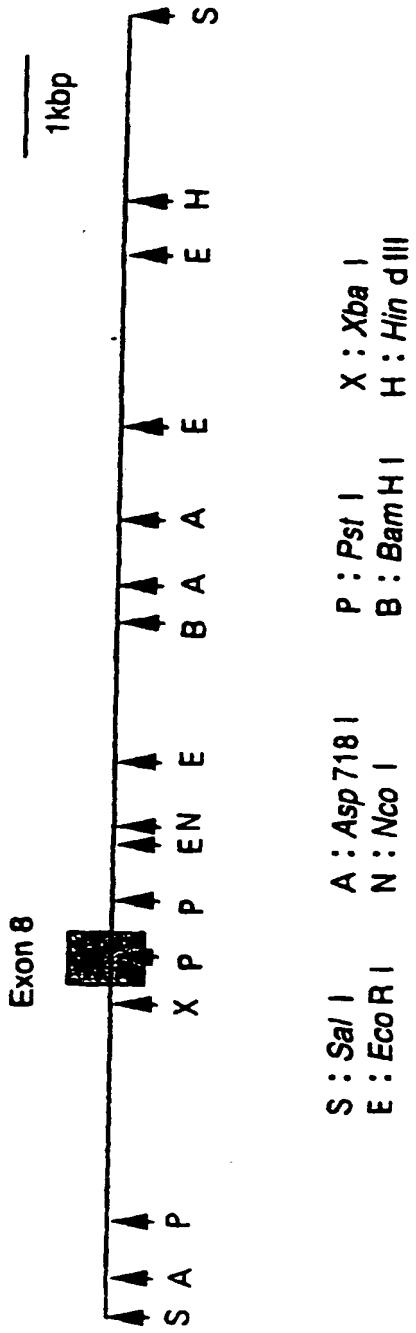
**[0038]** The gene-mutated animal of the present invention has a characteristic feature of producing amyloid β protein in a larger amount in comparison with a normal animal due to the genetic mutation. An increased amount of amyloid β protein achieved by the gene-mutated animal of the present invention is not particularly limited, and the amount may preferably be sufficient for recognition of a substantial difference in the evaluation of degrees of memory disorder, pathological observations, and various neural disorders as compared to a normal animal.

**[0039]** DNAs, plasmids, cell cultures, and embryos of mammalian cells provided by the present invention are characterized to have a mutant presenilin-1 gene and/or a mutant presenilin-2 gene. For example, a cDNA or a full-length chromosomal DNA of a mutant presenilin-1 gene encoding the mutant presenilin-1 protein, preferably an OS-2 type mutant presenilin-1 protein, or the DNA sequence comprising one or more mutation sites; a plasmid comprising a DNA being the above cDNA or full length chromosomal DNA, or the above DNA comprising one or more mutation sites, Which is further introduced with an Sau3AI site; a chromosomal DNA comprising exon 8 of a mutant presenilin-1 gene encoding an OS-2 type mutant presenilin-1 protein fall within the present invention. Further, the present invention encompasses the above gene or the DNA which further comprises one or more, preferably 1 to 20, more preferably 1 to several substitutions of bases.

**[0040]** Examples of DNAs and plasmids of the present invention include, for example:

1) a DNA comprising a mutant presenilin-1 gene encoding a mutant presenilin-1 protein wherein isoleucine at posi-

Fig. 1



presenilin-1 gene is introduced by homologous recombination, are examined. The preserved portion of the colony of the ES cells with the desired mutation introduced is taken and used in the process below.

**[0046]** From a pregnant mouse, an embryo at the 8-cell stage is removed. The embryo is sprinkled with about 20 of the above-mentioned preserved ES cells, and then introduced into the uterus of a pseudopregnant female mouse. From among the born young, mice of chimeric coat color are selected. The chimeric mouse is mated with a mouse C57BL/6 strain, and a mouse having the desired mutation can be obtained by the selection of those with agouti coat color from among the born young. The resulting mouse is heterozygote in relation to the presenilin-1 gene introduced with the mutation, whereas the presenilin-1 gene on the other chromosome is a wild type with no mutation.

**[0047]** As starting materials for preparing the probe for the cloning of the chromosomal DNA comprising exon 8 of the mouse presenilin-1 gene from the mouse genomic DNA library, a cDNA of a presenilin-1 gene, which is derived from a mammal other than mouse or human and whose nucleotide sequence has been known, may be used as well as those specifically mentioned in the Examples. As methods for obtaining the DNA fragment used as the probe, a method for a large scale preparation of a plasmid, which comprises a mouse chromosomal DNA comprising a region corresponding to exon 8 of the mouse presenilin-1 gene in chromosomal DNA, or a cDNA of a presenilin-1 gene derived from a mammal other than mouse or human or the like whose nucleotide sequence has been know, can be applied as well as amplification by PCR described in the Examples. Furthermore, after the plasmid is cleaved by restriction enzymes, a desired DNA fragment can be obtained by separating a portion used as the DNA fragment by means of agarose gel electrophoresis and the like.

**[0048]** As a method for labeling the DNA fragment, methods such as those utilizing PCR in the presence of $^{32}$P-dNTP may be used as well as the random priming method described in the Examples. Further, labeling may be introduced by PCR or random priming using a pre-labeled oligodeoxynucleotide as a primer. For the labeling, chemiluminescence using Biotin-Avidin or alkalinephosphatase or the like may also be used, as well as radioisotopes explained in the examples. An RNA fragment labeled by using T3 or T7 RNA polymerase may also be used as a probe. Various methods for preparing a probe are known other than those mentioned above, and a desired probe may be obtained by any method.

**[0049]** For introducing a desired mutation in a DNA, methods specifically described in the Examples can be applied. In addition, a plasmid derived from a bacteriophage such as M13 or a plasmid duplicated using ung$^-$ Escherichia coli is bound complimentarily with an oligodeoxynucleotide synthesized for introducing a mutation at a desired mutation site (bases of the site to be introduced with the mutation are not complimentary), and the resulting complex is used as a primer to prepare a heteroduplex DNA plasmid using a DNA polymerase, and then Escherichia coli (ung$^+$) is transformed with the resulting plasmid to obtain a plasmid having a desired mutation. Another method (cassette method) is applied for to obtain a plasmid having a desired mutation, which comprises the steps of synthesizing two oligodeoxynucleotide, which have modified bases to introduce a desired mutation, and are capable of annealing in a mutually complimentary manner and designed to give restriction enzyme sites at both terminals, and ligating the oligodeoxynucleotide to a plasmid for introduction of a mutation using DNA ligase. By appropriately modifying or altering the above methods depending on a purpose, the object may sometimes be more effectively achieved. In addition, as method for introducing a mutation, various methods available in the art are known, and accordingly, any method can be applied to achieve the object.

**[0050]** The targeting vector may preferably comprise a selective marker expression unit as an essential element which comprises a mouse chromosomal DNA fragment introduced with a mutation, a DNA fragment encoding a selective marker, a promoter for controlling transcription thereof, and a terminator. The mouse chromosomal DNA fragment introduced with a mutation is a necessary portion for causing homologous recombination in the ES cell, and the mouse chromosomal DNA fragments flanking the position of the mutation at both sides are also necessary. The target vector thus has a DNA fragment in which only the mutated bases are different from a native mouse chromosomal DNA. The length of the fragment may preferably about 10kbp, and generally some degree of lengthening or shortening is permissible. However, where the fragment is too short, frequency of homologous recombination may sometimes be lowered.

**[0051]** As selective markers, positive selective markers such as neomycin-resistant gene and hygromycin-resistant gene, and negative selective markers such as thymidine kinase gene of herpes simplex virus and fragment A of diphtheria toxin are known. Any of markers used for cell culture may be used in ES cells. Where a negative selective marker is used, it is necessary to insert the marker outside the mouse chromosomal DNA fragment of the targeting vector. Where a positive selective marker is used, it is necessary to insert the expression unit in an intron in the mouse chromosomal DNA fragment of the targeting vector. When a positive marker is inserted in an exon, the inserted gene generally loses function, and a mouse cannot be sometimes produced which is to be produced for examination of effects of the mutation as an ultimate purpose.

**[0052]** As an ES cell line, cell lines deriving from mouse 129 strain are frequently used. As ES cells deriving from the above mouse strain, ES cells such as D3, CCE, J1, and AB1 may be used as well as R1 described in the Examples. For example, mouse-derived ES cells such as from C57BL/6 mouse strain may also be used other than those from 129 strain. As methods for the introduction of the targeting vector into ES cells, electroporation as described in the Exam-

5'-TGTGGTCGGGATGAMCGCCACCCACTGGAAAGGCCC-3'
wherein M represents T or C.

25. A gene encoding a mouse mutant presenilin-1 protein wherein isoleucine at position 213 is substituted with an amino acid other than isoleucine in an amino acid sequence of a mouse presenilin-1 protein.

26. The gene according to claim 25, wherein the substitution is from isoleucine to threonine.

27. A plasmid comprising:

(1) a gene encoding a mouse mutant presenilin-1 protein wherein isoleucine at position 213 is substituted with an amino acid other than isoleucine in an amino acid sequence of a mouse presenilin-1 protein: and
(2) a neomycine expression unit flanked by loxPs.

28. The plasmid according to claim 27, wherein the substitution is from isoleucine to threonine.

29. An embryo introduced with a plasmid comprising a DNA represented by the nucleotide sequence:
5'-TGTGGTCGGGATGATMGCCACCCACTGGAAAGGCCC-3'
wherein M represents T or C.

30. An embryo obtained by homologous recombination using the plasmid according to any one of claims 20, 22, 23, 24, 27, or 28.

31. The embryo according to claim 29 or 30, wherein the embryo is derived from a mammalian rodent.

32. The embryo according to any one of claims 29 to 31, wherein the embryo is an embryonic stem cell derived from a mouse.

33. A primary cell culture or a subcultured cell obtainable by isolating a cell from the gene-mutated animal according to any one of claims 1 to 18 and culturing said cell by tissue culture.

34. A method for producing a non-human gene-mutated animal, wherein the method comprises the step of transferring a mutant presenilin-1 gene by homologous recombination into an embryo of an animal, wherein the mutant presenilin-1 gene is capable of expressing a mutant presenilin-1 protein and inducing production of amyloid β protein in an amount sufficient to form a progressive neural disease in the hippocampus or a peripheral portion of the cerebral cortex of the brain.

35. The method according to claim 35, wherein a mutant presenilin-1 protein is expressed in which isoleucine at position 213 is substituted with an amino acid other than isoleucine.

36. A method for evaluating a substance useful for therapeutic and/or preventive treatment of Alzheimer's disease which comprises the step of subjecting the gene-mutated animal according to any one of claims 1 to 18 which is administered with a test substance to a comparison with said gene-mutated animal not administered with the test substance.

37. The method for evaluation according to claim 36, wherein the comparison is conducted by using a memory learning test.

38. The method for evaluation according to claim 36, wherein the comparison is conducted by using a pathological test.

39. The method for evaluation according to claim 36, wherein the comparison is conducted by a pathological test based on neuropathology in a peripheral portion of the cerebral cortex.

40. The method for evaluation according to claim 38 or claim 39, wherein the comparison conducted by the pathological test based on neuropathology is a comparison of one or more items selected from the group consisting of suppression of decrease in overgrown gliosis in a peripheral portion of the cerebral cortex of the brain, suppression of decrease in uptake of 2-deoxyglucose in a peripheral portion of the cerebral cortex of the brain, and suppression of decrease in availability of 2-deoxyglucose in the cerebral cortex of the brain.

somal DNA with restriction enzyme, electrophoresis is conducted using agarose gel or acrylamide gel. The DNA is then blotted onto a membrane filter, and Southern blotting is performed using as a probe an oligodeoxynucleotide having a sequence which enables binding specifically to a gene encoding the APP mutant, and then density of the resulting bands are measured.

**[0061]** Similarly to the above process, possession of the mutant presenilin-1 gene of this invention in a homozygous state can be verified. Oligodeoxynucleotides used as probes in Southern blotting can be used after being labeled with means ordinarily used in Southern blotting such as a radioactive isotope and a fluorescent dye. A mouse having both of the gene encoding the APP mutation and the mutant presenilin-1 gene of the present invention can thus be produced. A hybrid mouse produced by the above method is characterized by higher productivity of amyloid β protein in the brain and promoted amyloid deposition.

**[0062]** Using the gene-mutated animal, the cells transferred with the mutant presenilin gene, the plasmid comprising the mutant presenilin gene and the like, it is possible to screen substances useful for preventive and/or therapeutic treatment of Alzheimer's disease and to evaluate their utility. Accumulation of amyloid β in a healthy mammal progresses very slowly, whereas the gene-mutated animal of the present invention has a characteristic feature of higher productivity of amyloid β. Therefore, by administering variety of test substances to the gene-mutated animal of the present invention, and comparing the animal with non-administered animals or animals administered with a control substance, it is possible to evaluate substances useful for preventive and/or therapeutic treatment of Alzheimer's disease. A typical example of the evaluation includes a screening of test substances, and conditions, pathological observations, pharmacological tests and the like can be applied as examinations.

**[0063]** Where the cells of the present invention are used, cells are isolated from the animal of the present invention for the use as a primary cell culture, and then the cells can be stabilized and made into a subcultured cell line by immortalizing the cells of primary culture by treatment with a virus or the like, subculturing the cells by isolating a portion of the culture and subjecting to further cultivation in a fresh tissue culture medium. The cells of the present invention encompass the primary cell culture such as nerve cells isolated from the gene-mutated animal, as well as subcultured cells, i.e., so-called cell lines, obtained by subculturing the primary culture. When a nerve cell is used as the cell of the present invention, the cell expresses a large amount of amyloid β protein due to a result of the expression of mutant presenilin-1 protein by the cell. Substance which prevent or delay the nerve cell death related to accumulation of amyloid β can be screened and utility thereof can also be evaluated by adding a test substance to an in vitro culture system of such nerve cells, and comparing, for example, cell survival period or surviving cell number after a certain period of time.

Examples

**[0064]** The present invention will be more specifically explained by way of examples. However, scope of the present invention is not limited to these examples. In the following examples, presenilin-1 gene is occasionally referred to as PS-1.

Example 1: Cloning of Chromosomal DNA containing Exon 8 of Mouse Presenilin-1 (PS-1) Gene

**[0065]** To construct a probe for isolating a chromosomal DNA containing exon 8 of the mouse PS-1 gene, the following two oligodeoxynucleotides were synthesized:

PR-8-U: 5'-GGAATTTTGGTGTGGTCGGGATGAT-3' (25-mer)
PR-8-L: 5'-GGTCCATTCGGGGAGGTACTTGA-3' (23-mer)

**[0066]** PCR was carried out by using these two oligodeoxynucleotides as PCR primers and DNA extracted from 129 SVJ mouse genomic library (Stratagene) to obtain amplified DNA fragment of approximately 130 bp. The fragment was then labeled by random priming method in the presence of $^{32}$P-dCTP and then used as probes for screening of the 129 SVJ mouse genomic library. The resulting positive phage clones were examined and confirmed that they carried the desired chromosomal DNA including exon 8 of the mouse PS-1 gene. The cloned chromosomal DNA was designated as Pα and subjected to restriction mapping (Figure 1).

Example 2: Construction of Plasmid for introducing Mutation

**[0067]** DNA was extracted from the cloned phage carrying Pα and cleaved with Sal I, and then subjected to electrophoresis on 1.0 % agarose gel to collect Pα. After the cleavage with Pst I and Xba I, the product was subjected to electrophoresis on 1% agarose gel to collect a DNA fragment of approximately 600 bp including a nucleotide sequence encoding isoleucine at position 213 of mouse PS-1. The resulting DNA fragment was designated as X-1. X-1 was

uation of usefulness thereof can be conducted by using the gene-mutated animal of the present invention.

## Claims

1. A non-human gene-mutated animal having a mutant presenilin-1 gene.

2. The gene-mutated animal according to claim 1, wherein the animal has a mutant presenilin-1 gene which comprises a DNA having a sequence encoding a presenilin-1 protein in which an amino acid in the amino acid sequence of the presenilin-1 protein is substituted with a different amino acid.

3. A non-human gene-mutated animal having a mutant presenilin-1 gene which comprises a DNA having a sequence encoding a mutant presenilin-1 protein which has an amino acid sequence in which one or more amino acids at positions selected from the group consisting of amino acid numbers 79, 82, 96, 115, 120, 135, 139, 143, 146, 163, 209, 213, 231, 235, 246, 250, 260, 263, 264, 267, 269, 280, 285, 286, 290, 318, 384, 392, 410, 426, and 436 is substituted with different amino acid(s) in the amino acid sequences of presenilin-1 protein.

4. A non-human gene-mutated animal having a mutant presenilin-1 gene which comprises a DNA having a sequence encoding a mutant presenilin-1 protein which has one or more mutations selected from the group consisting of A79V, V82L, V96F, Y115H, Y115C, E120K, E120D, N135D, M139V, M139T, M139I, I143F, I143T, M146L, M146V, H163Y, H163R, G209V, I213T, A231T, A231V, L235P, A246E, L250S, A260V, C263R, P264L, P267S, R269G, R269G, R269H, E280A, E280G, A285V, L286V, S290C, E318G, G384A, L392V, C410Y, A426P and P436S in the amino acid sequence of presenilin-1 protein, wherein each alphabet represents an amino acid expressed as a one-letter symbol, each number represents an amino acid number from the N-terminus of the presenilin-1 protein, and the descriptions mean that a wild-type amino acid shown in the left of the numerical figure is substituted with an amino acid shown in the right.

5. A non-human gene-mutated animal having a mutant presenilin-1 gene which comprises a DNA having a sequence encoding a mutant presenilin-1 protein in which isoleucine at position 213 of a presenilin-1 protein is substituted with an amino acid other than isoleucine.

6. A non-human gene-mutated animal having a mutant presenilin-1 gene which comprises a DNA having a sequence encoding a mutant presenilin-1 protein in which isoleucine at position 213 of a presenilin-1 protein is substituted with threonine.

7. The non-human gene-mutated animal according to any one of claims 1 to 6, wherein the animal has the mutant presenilin-1 gene wherein a DNA sequence encoding around an amino acid at position 213 in an amino acid sequence of the presenilin-1 protein is mutated to the following sequence:
5'-TGTGGTCGGGATGATMGCC ANC CACTGGAAAGGCCC-3'
wherein N represents a base other than T, M represents T or C, and the underlined bases encode the amino acid at position 213.

8. The non-human gene-mutated animal according to any one of claims 1 to 6, wherein the animal has the mutant presenilin-1 gene wherein a DNA sequence encoding around an amino acid at position 213 in an amino acid sequence of the presenilin-1 protein is mutated to the following sequence:
5'-TGTGGTCGGGATGATMGCC ANC CACTGGAAAGGCCC-3'
wherein N represents C, M represents T or C, and the underlined bases encode the amino acid at position 213.

9. The non-human gene-mutated animal according to any one of claims 1 to 6, wherein the animal has the mutant presenilin-1 gene wherein a DNA sequence encoding around an amino acid at position 213 in an amino acid sequence of the presenilin-1 protein is mutated to the following sequence:
5'-TGTGGTCGGGATGATMGCC XYZ CACTGGAAAGGCCC-3'
wherein XYZ represents a codon as triplet bases which encodes an amino acids other than isoleucine, M represents T or C, and the underlined bases encode the amino acid at position 213.

10. A non-human gene-mutated animal having a mutant presenilin-2 gene which comprises a DNA having a sequence encoding a protein in which an amino acid at position 141 and/or 436 is substituted with a different amino acid in an amino acid sequence of a presenilin-2 protein.

5'-TCTAGACGGCCGTCTAGA-3'
3'-AGATCTGCCGGCAGATCT-5'
Xba I Eag I Xba I

**[0075]** After the plasmid pmSB-0' was cleaved with Xba I, the above deoxynucleotide was added to the product and ligated using T4 DNA ligase, and then used to transform E. coli to obtain a plasmid pmSB-0'eag in which the Eag I site was inserted into the Xba I site of the plasmid pmSB-0'. After cleavage of pmSB-0'eag with Nco I and Sal I, resulting fragments were subjected to electrophoresis on 1% agarose gel to collect an approximately 5.3-kbp DNA fragment SN including exon 8. Separately, plasmid pSB-1 was cleaved with BamH I and Nco I and then subjected to electrophoresis on 1% agarose gel to collect an approximately 2-kbp DNA fragment NB not containing exon 8. The fragments SN and NB were ligated using T4 DNA ligase and treated with BamH I and Sal I to obtain a DNA fragment in which both DNA fragments were ligated at the Nco I site. This DNA fragment was further ligated to pBluescript II KS+ using T4 NDA ligase and then used to transform E. coli to obtain a plasmid pA (Figure 5), wherein the pBluescript II KS+ was cleaved beforehand with Not I, blunt-ended with mung bean nuclease, re-ligated using T4 DNA ligase to break the Not I site and the Eag I site overlapping with the site, and cleaved with BamH I and Sal I.

Example 6: Construction of Targeting Vector

**[0076]** Plasmid pPNT (Victor L. J. et al., Cell Vol. 65, p.1153, 1991) was cleaved with Xho I and BamH I and then treated with T4 DNA polymerase to form blunt ends and subjected to electrophoresis on 1 % agarose gel. The collected approximately 1.7-kbp DNA fragment containing a neo expression unit was ligated using T4 DNA ligase to the plasmid pBS246 (GIBCO BRL) which was cleaved beforehand with BamH I and treated with T4 DNA polymerase to form blunt ends, and then used to transform E. coli to obtain a plasmid pBS246neo. The plasmid was cleaved with Not I and then subjected to electrophoresis on 1% agarose gel to collect an approximately 2-kbp DNA fragment including the neo expression unit flanked by loxP sequences. The obtained DNA fragment was ligated using T4 DNA ligase to the plasmid pA which was cleaved beforehand with Eag I, and then used to transform E. coli. Colonies of transformed cells were screened to obtain plasmid pB in which the neo gene and the PS-1 gene were oriented in the same direction (Figure 6).

**[0077]** After cleavage of the plasmid pB with BamH I and Sal I, the resulting fragments were subjected to electrophoresis on 1% agarose gel to collect a DNA fragment C containing the OS-2 type mutation and the neo expression unit flanked by loxP sequences. Similarly, Pα was cleaved with Sal I and BamH I, and the resulting DNA fragment of approximately 6.5 kbp was subcloned into pBluescript II KS+ to construct a plasmid pSB-2, which was then cleaved with Hind III and BamH I and subjected to electrophoresis on 1% agarose gel to collect a DNA fragment D of approximately 4kbp. DNA fragments C and D were ligated using T4 DNA ligase, and then the product was cleaved with Hind III and Sal I to obtain a DNA fragment in which C and D were ligated at the BamH I site. The obtained DNA fragment was further ligated using T4 DNA ligase to the pBluescript II KS+ which was cleaved beforehand with Hind III and Sal I, and then used to transform E. coli to obtain a targeting vector pOS-2neoloxP (Figure 7).

Example 7: Introduction of Targeting Vector into ES Cells

**[0078]** Hereinafter in the examples, culture was carried out in an incubator at 37 °C under 5% $CO_2$. The targeting vector was introduced by electroporation into ES cells (R1) which were maintained in DMEM medium supplemented with 15% FBS and $10^3$ units/ml LIF (ESGRO) (the DMEM medium is hereinafter abbreviated as ES medium). Culture medium was replaced with fresh ES medium one day before electroporation, and the R1 cells were collected and washed with electroporation buffer (20 mM HEPES, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM $Na_2HPO_4$, 6 mM dextrose). R1 cells ($10^7$ cells) were mixed with 25 μg of the targeting vector pOS-2neoloxP, which was linearized using Not I, and 0.8 ml electroporation buffer in an electroporation cuvette. After 1 to 2 minutes, pulses were applied to the cells using Bio-Rad GenePulser (Bio-Rad) under pulse conditions of 240 V and 500 μF. The ES cells were collected by centrifugation and suspended in 30 ml ES medium. The ES cell suspension (2 ml) was put in each 10ml culture dish in which feeder cells were put in 8 ml ES medium. G418 (titer, 150 μg/ml) was added to the culture after 12 to 18 hours, followed by one-week culture. As the feeder cell, a fibroblast established by the present inventors was used which was isolated from an embryo of 12 to 13 days obtained by mating a HS1 knockout male mouse (I. Taniuchi et al., EMBO J. vol. 14, p. 3664. 1995) with an ICR female mouse of wild-type.

5'-TCTAGACGGCCGTCTAGA-3'
3'-AGATCTGCCGGCAGATCT-5'
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## Claims

1. A non-human gene-mutated animal having a mutant presenilin-1 gene.

2. The gene-mutated animal according to claim 1, wherein the animal has a mutant presenilin-1 gene which comprises a DNA having a sequence encoding a presenilin-1 protein in which an amino acid in the amino acid sequence of the presenilin-1 protein is substituted with a different amino acid.

3. A non-human gene-mutated animal having a mutant presenilin-1 gene which comprises a DNA having a sequence encoding a mutant presenilin-1 protein which has an amino acid sequence in which one or more amino acids at positions selected from the group consisting of amino acid numbers 79, 82, 96, 115, 120, 135, 139, 143, 146, 163, 209, 213, 231, 235, 246, 250, 260, 263, 264, 267, 269, 280, 285, 286, 290, 318, 384, 392, 410, 426, and 436 is substituted with different amino acid(s) in the amino acid sequences of presenilin-1 protein.

4. A non-human gene-mutated animal having a mutant presenilin-1 gene which comprises a DNA having a sequence encoding a mutant presenilin-1 protein which has one or more mutations selected from the group consisting of A79V, V82L, V96F, Y115H, Y115C, E120K, E120D, N135D, M139V, M139T, M139I, I143F, I143T, M146L, M146V, H163Y, H163R, G209V, I213T, A231T, A231V, L235P, A246E, L250S, A260V, C263R, P264L, P267S, R269G, R269G, R269H, E280A, E280G, A285V, L286V, S290C, E318G, G384A, L392V, C410Y, A426P and P436S in the amino acid sequence of presenilin-1 protein, wherein each alphabet represents an amino acid expressed as a one-letter symbol, each number represents an amino acid number from the N-terminus of the presenilin-1 protein, and the descriptions mean that a wild-type amino acid shown in the left of the numerical figure is substituted with an amino acid shown in the right.

5. A non-human gene-mutated animal having a mutant presenilin-1 gene which comprises a DNA having a sequence encoding a mutant presenilin-1 protein in which isoleucine at position 213 of a presenilin-1 protein is substituted with an amino acid other than isoleucine.

6. A non-human gene-mutated animal having a mutant presenilin-1 gene which comprises a DNA having a sequence encoding a mutant presenilin-1 protein in which isoleucine at position 213 of a presenilin-1 protein is substituted with threonine.

7. The non-human gene-mutated animal according to any one of claims 1 to 6, wherein the animal has the mutant presenilin-1 gene wherein a DNA sequence encoding around an amino acid at position 213 in an amino acid sequence of the presenilin-1 protein is mutated to the following sequence:
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wherein N represents a base other than T, M represents T or C, and the underlined bases encode the amino acid at position 213.

8. The non-human gene-mutated animal according to any one of claims 1 to 6, wherein the animal has the mutant presenilin-1 gene wherein a DNA sequence encoding around an amino acid at position 213 in an amino acid sequence of the presenilin-1 protein is mutated to the following sequence:
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9. The non-human gene-mutated animal according to any one of claims 1 to 6, wherein the animal has the mutant presenilin-1 gene wherein a DNA sequence encoding around an amino acid at position 213 in an amino acid sequence of the presenilin-1 protein is mutated to the following sequence:
5'-TGTGGTCGGGATGATMGCC XYZ CACTGGAAAGGCCC-3'
wherein XYZ represents a codon as triplet bases which encodes an amino acids other than isoleucine, M represents T or C, and the underlined bases encode the amino acid at position 213.

10. A non-human gene-mutated animal having a mutant presenilin-2 gene which comprises a DNA having a sequence encoding a protein in which an amino acid at position 141 and/or 436 is substituted with a different amino acid in an amino acid sequence of a presenilin-2 protein.

somal DNA with restriction enzyme, electrophoresis is conducted using agarose gel or acrylamide gel. The DNA is then blotted onto a membrane filter, and Southern blotting is performed using as a probe an oligodeoxynucleotide having a sequence which enables binding specifically to a gene encoding the APP mutant, and then density of the resulting bands are measured.

**[0061]** Similarly to the above process, possession of the mutant presenilin-1 gene of this invention in a homozygous state can be verified. Oligodeoxynucleotides used as probes in Southern blotting can be used after being labeled with means ordinarily used in Southern blotting such as a radioactive isotope and a fluorescent dye. A mouse having both of the gene encoding the APP mutation and the mutant presenilin-1 gene of the present invention can thus be produced. A hybrid mouse produced by the above method is characterized by higher productivity of amyloid β protein in the brain and promoted amyloid deposition.

**[0062]** Using the gene-mutated animal, the cells transferred with the mutant presenilin gene, the plasmid comprising the mutant presenilin gene and the like, it is possible to screen substances useful for preventive and/or therapeutic treatment of Alzheimer's disease and to evaluate their utility. Accumulation of amyloid β in a healthy mammal progresses very slowly, whereas the gene-mutated animal of the present invention has a characteristic feature of higher productivity of amyloid β. Therefore, by administering variety of test substances to the gene-mutated animal of the present invention, and comparing the animal with non-administered animals or animals administered with a control substance, it is possible to evaluate substances useful for preventive and/or therapeutic treatment of Alzheimer's disease. A typical example of the evaluation includes a screening of test substances, and conditions, pathological observations, pharmacological tests and the like can be applied as examinations.

**[0063]** Where the cells of the present invention are used, cells are isolated from the animal of the present invention for the use as a primary cell culture, and then the cells can be stabilized and made into a subcultured cell line by immortalizing the cells of primary culture by treatment with a virus or the like, subculturing the cells by isolating a portion of the culture and subjecting to further cultivation in a fresh tissue culture medium. The cells of the present invention encompass the primary cell culture such as nerve cells isolated from the gene-mutated animal, as well as subcultured cells, i.e., so-called cell lines, obtained by subculturing the primary culture. When a nerve cell is used as the cell of the present invention, the cell expresses a large amount of amyloid β protein due to a result of the expression of mutant presenilin-1 protein by the cell. Substance which prevent or delay the nerve cell death related to accumulation of amyloid β can be screened and utility thereof can also be evaluated by adding a test substance to an in vitro culture system of such nerve cells, and comparing, for example, cell survival period or surviving cell number after a certain period of time.

Examples

**[0064]** The present invention will be more specifically explained by way of examples. However, scope of the present invention is not limited to these examples. In the following examples, presenilin-1 gene is occasionally referred to as PS-1.

Example 1: Cloning of Chromosomal DNA containing Exon 8 of Mouse Presenilin-1 (PS-1) Gene

**[0065]** To construct a probe for isolating a chromosomal DNA containing exon 8 of the mouse PS-1 gene, the following two oligodeoxynucleotides were synthesized:

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**[0066]** PCR was carried out by using these two oligodeoxynucleotides as PCR primers and DNA extracted from 129 SVJ mouse genomic library (Stratagene) to obtain amplified DNA fragment of approximately 130 bp. The fragment was then labeled by random priming method in the presence of $^{32}$P-dCTP and then used as probes for screening of the 129 SVJ mouse genomic library. The resulting positive phage clones were examined and confirmed that they carried the desired chromosomal DNA including exon 8 of the mouse PS-1 gene. The cloned chromosomal DNA was designated as Pα and subjected to restriction mapping (Figure 1).

Example 2: Construction of Plasmid for introducing Mutation

**[0067]** DNA was extracted from the cloned phage carrying Pα and cleaved with Sal I, and then subjected to electrophoresis on 1.0 % agarose gel to collect Pα. After the cleavage with Pst I and Xba I, the product was subjected to electrophoresis on 1% agarose gel to collect a DNA fragment of approximately 600 bp including a nucleotide sequence encoding isoleucine at position 213 of mouse PS-1. The resulting DNA fragment was designated as X-1. X-1 was

5'-TGTGGTCGGGATGAMCGCCACCCACTGGAAAGGCCC-3'
wherein M represents T or C.

25. A gene encoding a mouse mutant presenilin-1 protein wherein isoleucine at position 213 is substituted with an amino acid other than isoleucine in an amino acid sequence of a mouse presenilin-1 protein.

26. The gene according to claim 25, wherein the substitution is from isoleucine to threonine.

27. A plasmid comprising:

(1) a gene encoding a mouse mutant presenilin-1 protein wherein isoleucine at position 213 is substituted with an amino acid other than isoleucine in an amino acid sequence of a mouse presenilin-1 protein: and
(2) a neomycine expression unit flanked by loxPs.

28. The plasmid according to claim 27, wherein the substitution is from isoleucine to threonine.

29. An embryo introduced with a plasmid comprising a DNA represented by the nucleotide sequence:
5'-TGTGGTCGGGATGATMGCCACCCACTGGAAAGGCCC-3'
wherein M represents T or C.

30. An embryo obtained by homologous recombination using the plasmid according to any one of claims 20, 22, 23, 24, 27, or 28.

31. The embryo according to claim 29 or 30, wherein the embryo is derived from a mammalian rodent.

32. The embryo according to any one of claims 29 to 31, wherein the embryo is an embryonic stem cell derived from a mouse.

33. A primary cell culture or a subcultured cell obtainable by isolating a cell from the gene-mutated animal according to any one of claims 1 to 18 and culturing said cell by tissue culture.

34. A method for producing a non-human gene-mutated animal, wherein the method comprises the step of transferring a mutant presenilin-1 gene by homologous recombination into an embryo of an animal, wherein the mutant presenilin-1 gene is capable of expressing a mutant presenilin-1 protein and inducing production of amyloid β protein in an amount sufficient to form a progressive neural disease in the hippocampus or a peripheral portion of the cerebral cortex of the brain.

35. The method according to claim 35, wherein a mutant presenilin-1 protein is expressed in which isoleucine at position 213 is substituted with an amino acid other than isoleucine.

36. A method for evaluating a substance useful for therapeutic and/or preventive treatment of Alzheimer's disease which comprises the step of subjecting the gene-mutated animal according to any one of claims 1 to 18 which is administered with a test substance to a comparison with said gene-mutated animal not administered with the test substance.

37. The method for evaluation according to claim 36, wherein the comparison is conducted by using a memory learning test.

38. The method for evaluation according to claim 36, wherein the comparison is conducted by using a pathological test.

39. The method for evaluation according to claim 36, wherein the comparison is conducted by a pathological test based on neuropathology in a peripheral portion of the cerebral cortex.

40. The method for evaluation according to claim 38 or claim 39, wherein the comparison conducted by the pathological test based on neuropathology is a comparison of one or more items selected from the group consisting of suppression of decrease in overgrown gliosis in a peripheral portion of the cerebral cortex of the brain, suppression of decrease in uptake of 2-deoxyglucose in a peripheral portion of the cerebral cortex of the brain, and suppression of decrease in availability of 2-deoxyglucose in the cerebral cortex of the brain.

presenilin-1 gene is introduced by homologous recombination, are examined. The preserved portion of the colony of the ES cells with the desired mutation introduced is taken and used in the process below.

**[0046]** From a pregnant mouse, an embryo at the 8-cell stage is removed. The embryo is sprinkled with about 20 of the above-mentioned preserved ES cells, and then introduced into the uterus of a pseudopregnant female mouse. From among the born young, mice of chimeric coat color are selected. The chimeric mouse is mated with a mouse C57BL/6 strain, and a mouse having the desired mutation can be obtained by the selection of those with agouti coat color from among the born young. The resulting mouse is heterozygote in relation to the presenilin-1 gene introduced with the mutation, whereas the presenilin-1 gene on the other chromosome is a wild type with no mutation.

**[0047]** As starting materials for preparing the probe for the cloning of the chromosomal DNA comprising exon 8 of the mouse presenilin-1 gene from the mouse genomic DNA library, a cDNA of a presenilin-1 gene, which is derived from a mammal other than mouse or human and whose nucleotide sequence has been known, may be used as well as those specifically mentioned in the Examples. As methods for obtaining the DNA fragment used as the probe, a method for a large scale preparation of a plasmid, which comprises a mouse chromosomal DNA comprising a region corresponding to exon 8 of the mouse presenilin-1 gene in chromosomal DNA, or a cDNA of a presenilin-1 gene derived from a mammal other than mouse or human or the like whose nucleotide sequence has been know, can be applied as well as amplification by PCR described in the Examples. Furthermore, after the plasmid is cleaved by restriction enzymes, a desired DNA fragment can be obtained by separating a portion used as the DNA fragment by means of agarose gel electrophoresis and the like.

**[0048]** As a method for labeling the DNA fragment, methods such as those utilizing PCR in the presence of $^{32}$P-dNTP may be used as well as the random priming method described in the Examples. Further, labeling may be introduced by PCR or random priming using a pre-labeled oligodeoxynucleotide as a primer. For the labeling, chemiluminescence using Biotin-Avidin or alkalinephosphatase or the like may also be used, as well as radioisotopes explained in the examples. An RNA fragment labeled by using T3 or T7 RNA polymerase may also be used as a probe. Various methods for preparing a probe are known other than those mentioned above, and a desired probe may be obtained by any method.

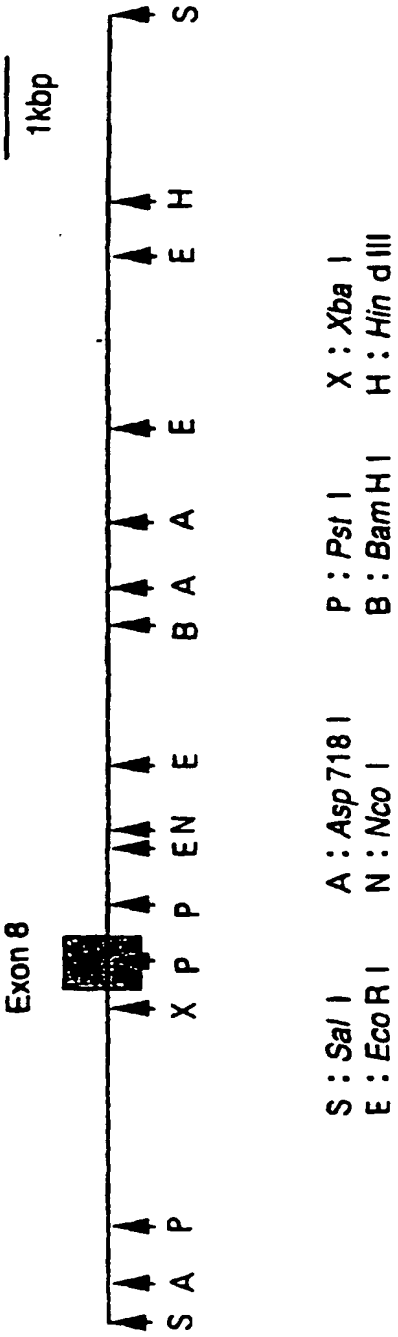
**[0049]** For introducing a desired mutation in a DNA, methods specifically described in the Examples can be applied. In addition, a plasmid derived from a bacteriophage such as M13 or a plasmid duplicated using ung$^-$ Escherichia coli is bound complimentarily with an oligodeoxynucleotide synthesized for introducing a mutation at a desired mutation site (bases of the site to be introduced with the mutation are not complimentary), and the resulting complex is used as a primer to prepare a heteroduplex DNA plasmid using a DNA polymerase, and then Escherichia coli (ung$^+$) is transformed with the resulting plasmid to obtain a plasmid having a desired mutation. Another method (cassette method) is applied for to obtain a plasmid having a desired mutation, which comprises the steps of synthesizing two oligodeoxynucleotide, which have modified bases to introduce a desired mutation, and are capable of annealing in a mutually complimentary manner and designed to give restriction enzyme sites at both terminals, and ligating the oligodeoxynucleotide to a plasmid for introduction of a mutation using DNA ligase. By appropriately modifying or altering the above methods depending on a purpose, the object may sometimes be more effectively achieved. In addition, as method for introducing a mutation, various methods available in the art are known, and accordingly, any method can be applied to achieve the object.

**[0050]** The targeting vector may preferably comprise a selective marker expression unit as an essential element which comprises a mouse chromosomal DNA fragment introduced with a mutation, a DNA fragment encoding a selective marker, a promoter for controlling transcription thereof, and a terminator. The mouse chromosomal DNA fragment introduced with a mutation is a necessary portion for causing homologous recombination in the ES cell, and the mouse chromosomal DNA fragments flanking the position of the mutation at both sides are also necessary. The target vector thus has a DNA fragment in which only the mutated bases are different from a native mouse chromosomal DNA. The length of the fragment may preferably about 10kbp, and generally some degree of lengthening or shortening is permissible. However, where the fragment is too short, frequency of homologous recombination may sometimes be lowered.

**[0051]** As selective markers, positive selective markers such as neomycin-resistant gene and hygromycin-resistant gene, and negative selective markers such as thymidine kinase gene of herpes simplex virus and fragment A of diphtheria toxin are known. Any of markers used for cell culture may be used in ES cells. Where a negative selective marker is used, it is necessary to insert the marker outside the mouse chromosomal DNA fragment of the targeting vector. Where a positive selective marker is used, it is necessary to insert the expression unit in an intron in the mouse chromosomal DNA fragment of the targeting vector. When a positive marker is inserted in an exon, the inserted gene generally loses function, and a mouse cannot be sometimes produced which is to be produced for examination of effects of the mutation as an ultimate purpose.

**[0052]** As an ES cell line, cell lines deriving from mouse 129 strain are frequently used. As ES cells deriving from the above mouse strain, ES cells such as D3, CCE, J1, and AB1 may be used as well as R1 described in the Examples. For example, mouse-derived ES cells such as from C57BL/6 mouse strain may also be used other than those from 129 strain. As methods for the introduction of the targeting vector into ES cells, electroporation as described in the Exam-

Fig. 1



mutant presenilin-1 protein or a mutant presenilin-2 protein. The mutant presenilin gene has the property of increasing the production of amyloid β protein. The gene-mutated animal of the present invention is a mammal transferred with the above-mentioned mutant presenilin gene for example by homologous recombination. The mutation existing in the mutant protein is preferably a result of substitution of an amino acid residue. The number of mutations is not limited, and may preferably be 1.

**[0033]** The full length sequence of a mammal-derived preselin-1 protein is described in, for example, E. Levy-Lahad, et al., Science, 269, pp.973-977, 1995. The full-length sequences of human and mouse presenilin-1 proteins and examples of DNA sequences that encode the proteins are shown in the sequence listings as SEQ ID NOS: 1 to 4. For example, in the mouse-derived presenilin-1, mutation sites may preferably be one or more sites selected from No. 79, No. 82, No. 96, No. 115, No. 120, No. 135, No. 139, No. 143, No. 146, No. 163, No. 209, No. 213, No. 231, No. 235, No. 246, No. 250, No. 260, No. 263, No. 264, No. 267, No. 269, No. 280, No. 285, No. 286, No. 290, No. 318, No. 384, No. 392, No. 410, No. 426, and No. 436.

**[0034]** More preferable mutations are one or more mutations selected from the group consisting of A79V, V82L, V96F, Y115H, Y115C, E120K, E120D, N135D, M139V, M139T, M139I, I143F, I143T, M146L, M146V, H163Y, H163R, G209V, I213T, A231T, A231V, L235P, A246E, L250S, A260V, C263R, P264L, P267S, R269G, R269G, R269H, E280A, E280G, A285V, L286V, S290C, E318G, G384A, L392V, C410Y, A426P, and P436S in the amino acid sequence of the presenilin-1 protein, more preferably in the amino acid sequence of the mouse-derived presenilin-1 protein. Among these mutations, the mutation wherein the amino acid at position 213 is substituted with another amino acid (referred to in some cases as "OS-2 type mutation" in the specification) is a particularly preferable mutation. For example, a mutation wherein isoleucine at position 213 is substituted with an amino acid other than isoleucine, or a mutation wherein isoleucine at position 213 is substituted with threonine is most preferable.

**[0035]** The full-length sequence of a mammal-derived preseline-2 protein is described in, for example, Science, 269, pp. 973-977, 1995. Position 141 and/or position 436 are preferable mutation sites, and in the mouse-derived sequence N141I and/or M239V are more preferable. One or more mutations may exist in either of presenilin-1 protein or presenilin-2 protein, or both of the proteins.

**[0036]** The gene-mutated animal of the present invention is characterized by having the above mutant presenilin-1 gene and/or mutant presenilin-2 gene on its chromosomal DNA. The gene-mutated animal is not limited so far that the animal is a mammal and a kind of the animal is not particularly limited. For example, a rodent may suitably be used. A mouse is particularly preferred. The gene-mutated animal of the present invention can be produced by constructing a plasmid using a DNA having a sequence of about 10kbp comprising a mutant presenilin gene, and then transferring the plasmid into an embryonic stem cell and thereby causing homologous recombination intracellularly.

**[0037]** The gene-mutated animal of the present invention is characterized in that the amino acid mutation occurs mostly at only one position due to the transfer of the aforementioned mutant presenilin-1 and/or presenilin-2 gene by homologous recombination. In the case of a so-called "trans-genic animal", a DNA sequence comprising a mutant portion is inserted randomly into chromosomal DNA, and tens of copies of a repeated sequence are inserted at plural sites. The gene-mutated animal of the present invention can avoid the problems, and it is possible to accurately analyze pathology of Alzheimer's disease at genetic level. Where a DNA comprises a marker or the like is transferred to the gene-mutated animal of the present invention, the animal may have a site of the marker and a sequence for insertion of the market For example, for insertion at a site capable of being cleaved with Sau3AI, one nucleotide can be substituted, and such substitution can be verified by cleaving a PCR product with Sau3AI, followed by subjecting the fragments to electrophoresis or the like.

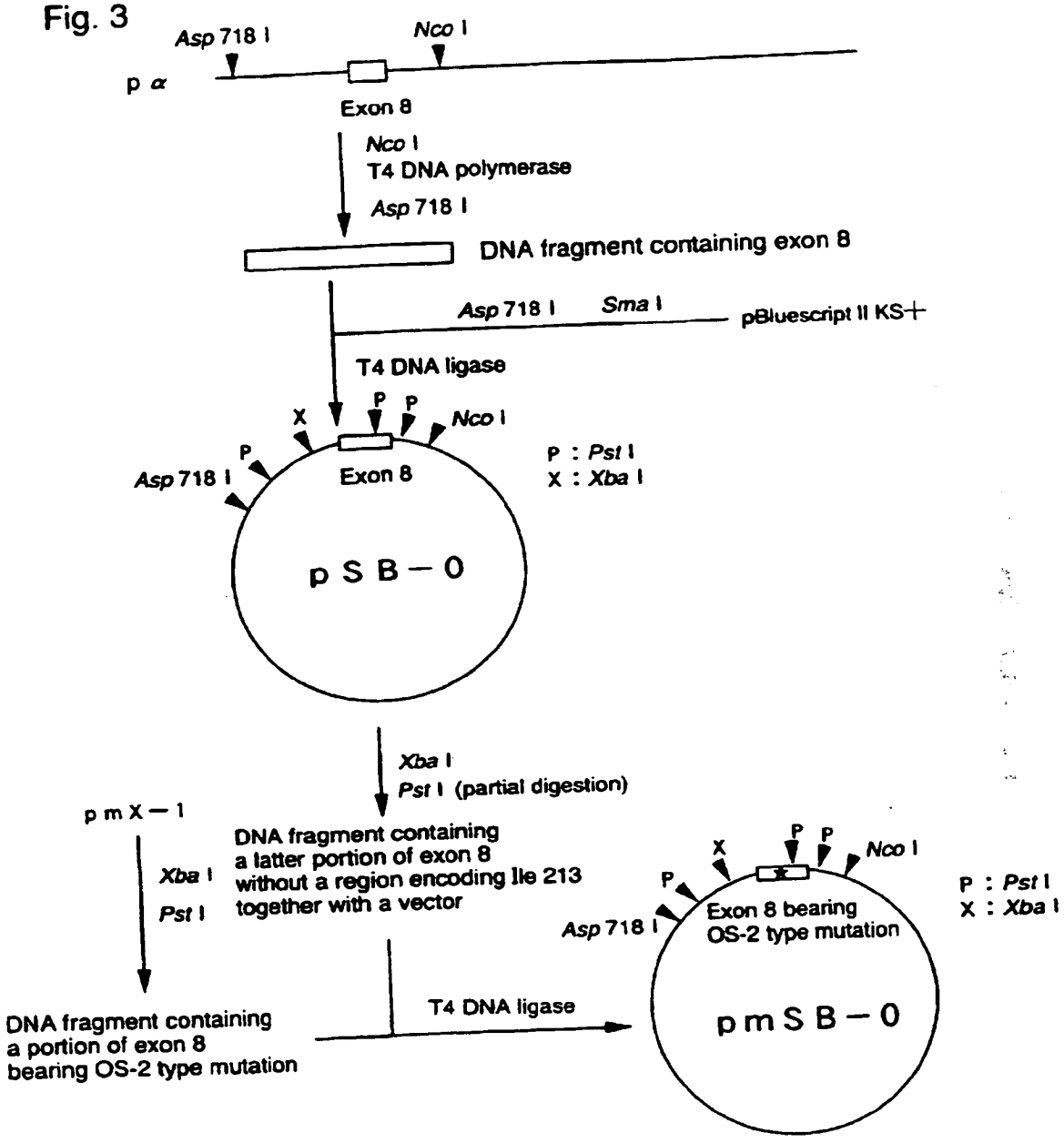
**[0038]** The gene-mutated animal of the present invention has a characteristic feature of producing amyloid β protein in a larger amount in comparison with a normal animal due to the genetic mutation. An increased amount of amyloid β protein achieved by the gene-mutated animal of the present invention is not particularly limited, and the amount may preferably be sufficient for recognition of a substantial difference in the evaluation of degrees of memory disorder, pathological observations, and various neural disorders as compared to a normal animal.

**[0039]** DNAs, plasmids, cell cultures, and embryos of mammalian cells provided by the present invention are characterized to have a mutant presenilin-1 gene and/or a mutant presenilin-2 gene. For example, a cDNA or a full-length chromosomal DNA of a mutant presenilin-1 gene encoding the mutant presenilin-1 protein, preferably an OS-2 type mutant presenilin-1 protein, or the DNA sequence comprising one or more mutation sites; a plasmid comprising a DNA being the above cDNA or full length chromosomal DNA, or the above DNA comprising one or more mutation sites, Which is further introduced with an Sau3AI site; a chromosomal DNA comprising exon 8 of a mutant presenilin-1 gene encoding an OS-2 type mutant presenilin-1 protein fall within the present invention. Further, the present invention encompasses the above gene or the DNA which further comprises one or more, preferably 1 to 20, more preferably 1 to several substitutions of bases.

**[0040]** Examples of DNAs and plasmids of the present invention include, for example:

1) a DNA comprising a mutant presenilin-1 gene encoding a mutant presenilin-1 protein wherein isoleucine at posi-

Fig. 3



a mutation of N141I and/or M239V in an amino acid sequence of a presenilin-2 protein.

**[0023]** As preferred embodiments of the aforementioned gene-mutated animals, the present invention provides the aforementioned gene-mutated animal wherein overexpression of amyloid β protein is caused by the mutant presenilin-1 gene and/or the mutant presenilin-2 gene; the aforementioned gene-mutated animal which can express the mutant presenilin protein and wherein the expression of said protein induces the production of amyloid β protein in an amount sufficient to form a progressive neural disease in a peripheral portion of the cerebral cortex of the brain of the animal; the aforementioned gene-mutated animal wherein the animal is a rodent, preferably a mouse; the aforementioned gene-mutated animal wherein the aforementioned mutant presenilin-1 gene and/or the aforementioned mutant presenilin-2 gene are transferred by homologous recombination; the aforementioned gene-mutated animal wherein amount of the amyloid protein expression in a brain tissue induced by the aforementioned presenilin-1 gene is sufficient to cause affected behavior in a memory learning test in comparison with a normal animal, and to induce abnormal neuropathy in a peripheral portion of the cerebral cortex of the hippocampus of the brain of the animal; and the non-human gene-mutated animal having a DNA which comprises a mutant preceilin-1 gene encoding a mutant preceilin-1 protein in which one or two or more amino acids is substituted with a different amino acid in the amino acid sequence of the presenilin-1 protein together with a DNA having a nucleotide sequence encoding a marker protein.

**[0024]** From further aspect, the present invention provides a plasmid comprising a DNA having a sequence of a mutant presenilin-1 gene wherein a DNA sequence encoding around an amino acid at position 213 of a presenilin-1 protein is the following sequence:

5'-TGTGGTCGGGATGATMGCC ANC CACTGGAAAGGCCC-3'

wherein N represents A, G, or C, M represents T or C, and the underlined bases encode an amino acid at position 213; and

a plasmid comprising a DNA having a sequence of a mutant presenilin-1 gene which encodes a mutant presenilin-1 protein wherein an amino acid at position 213 is substituted with an amino acid other than isoleucine in an amino acid sequence of the presenilin-1 protein and a DNA sequence encoding around the amino acid at position 213 of presenilin-1 protein is the following sequence:

5'-TGTGGTCGGGATGATMGCC XYZ CACTGGAAAGGCCC-3'

wherein M represents T or C, XYZ denotes a codon as triplet bases encoding an amino acid other than isoleucine, and the underlined bases encode the amino acid at position 213. Additionally, the present invention also provides a chromosomal DNA containing exon 8 of a mutant presenilin-1 gene encoding a mutant presenilin-1 protein wherein an amino acid at position 213 is substituted with an amino acid other than isoleucine in an amino acid sequence of a presenilin-1 protein.

**[0025]** Furthermore, the present invention provides a plasmid comprising a DNA wherein a Sau3AI site is introduced into a nucleotide sequence comprising the whole or a mutated part of a cDNA or chromosomal DNA of a mutant presenilin-1 gene encoding a mutant presenilin-1 protein in which an amino acid at position 213 is substituted with an amino acid other than isoleucine in an amino acid sequence of presenilin-1 protein. Also provided are the aforementioned plasmid wherein the substitution of the amino acid is isoleucine at position 213 with threonine; and a plasmid comprising a DNA specified by the following nucleotide sequence:

5'-TGTGGTCGGGATGATMGCCACCCACTGGAAAGGCCC-3'

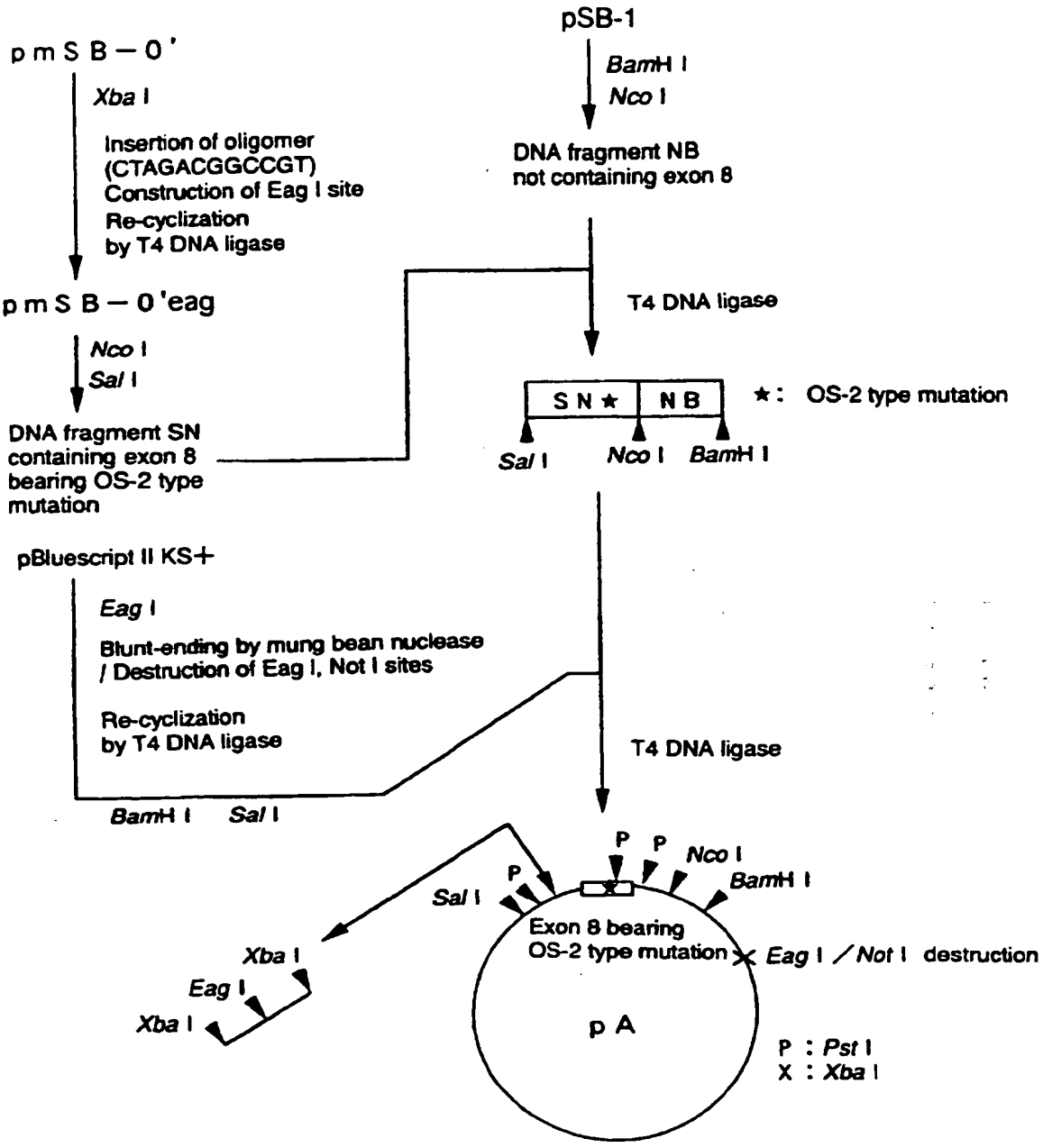
wherein M represents T or C.

**[0026]** In addition to the above inventions, the present invention also provides a gene encoding a mouse mutant presenilin-1 protein wherein isoleucine at position 213 is substituted with an amino acid other than isoleucine in an amino acid sequence of a mouse presenilin-1 protein; and the aforementioned gene wherein the substitution is from isoleucine to threonine. Also provided are a plasmid comprising: (1) a gene encoding a mouse mutant presenilin-1 protein wherein isoleucine at position 213 is substituted with an amino acid other than isoleucine in an amino acid sequence of a mouse presenilin-1 protein; and (2) a neomycine expression unit flanked by loxPs; and the aforementioned plasmid wherein the substitution is from isoleucine to threonine (loxP has been disclosed in Japanese Patent Laid-Open Publication (Kohyo) No. 4-501501, page4).

**[0027]** From further aspect, the present invention provides an embryo introduced with a plasmid comprising a DNA represented by the nucleotide sequence:

5'-TGTGGTCGGGATGATMGCCACCCACTGGAAAGGCCC-3' wherein M represents T or C; an embryo obtained by homologous recombination using each of the aforementioned plasmids; and the aforementioned embryo derived from a mammalian rodent, more preferably from a mouse. The invention also provides a primary cell culture or subcultured cell obtained by isolating a cell from the aforementioned gene-mutated animal and culturing the cell by tissue culture; a method for producing a non-human gene-mutated animal wherein the method comprises the step of transferring a mutant presenilin-1 gene by homologous recombination into an embryo of an animal, wherein the mutant presenilin-1 gene is capable of expressing the mutant presenilin-1 and inducing production of amyloid β protein in an amount sufficient to form a progressive neural disease in a peripheral portion of the cerebral cortex of the brain; and the aforementioned production method wherein a mutant presenilin-1 protein can be expressed wherein isoleucine at position 213 is

Fig. 5



gested to possibly participate in a certain step of intercellular signal transduction.

**[0010]** The first report on presenilin-1 protein describes that mutations causing the familial Alzheimer's disease are substitutions of amino acid residues at five positions. After this report, genes mutated at various sites were found from many families afflicted with familial Alzheimer's disease, which include OS-2 (isoleucine at position 213 is mutated to threonine) and OS-3 (valine at position 96 is mutated to phenylalanine), both reported by the present inventors (Kamino K. et al., Neurosci., Lett., Vol. 208, P195, 1996), and more than 40 types of amino acid substitutions have been known at more than 30 sites so far (Hardy. TINS, Vol. 20, P154, 1997).

**[0011]** At present, 70-80 % of the familial Alzheimer's disease is believed to be related to the mutation of presenilin-1 protein. Mutations at two sites have been reported as for presenilin-2 protein. As explained above, genetic analysis has proved that mutants of presenilin-1 and presenilin-2 proteins are deeply involved in the familial Alzheimer's disease.

**[0012]** Studies on mechanism how the mutants of presenilin-1 and presenilin-2 proteins cause the onset of Alzheimer's disease have also been progressed. It has been reported that Aβ 40 is almost the same level as normal presenilin-1 and presenilin-2 proteins, whilst Aβ 42 is highly increased as compared to normal presenilin-1 and presenilin-2 proteins in serum or a culture medium of dermal fibroblasts from a patient with Alzheimer's disease having the aforementioned mutants (Scheuner D. et al.: Nature Med., Vol. 2, P864, 1996); in a culture medium of a cell line transformed by mutants of presenilin-1 protein and presenilin-2 protein (Xia W et al.: J. Biol. Chem. Vol. 272, P7977, 1997; Borchelt D.R. et al.: Neuron, Vol. 17, P1005, 1996; Citron, M. et al. Nature Med., Vol. 3, P67, 1997); and in the brain tissue of a patient with familial Alzheimer's disease having the mutant presenilin-1 protein (Lemere C.A. et al.: Nature Med., Vol. 2., P1146, 1996).

**[0013]** These reports show that the mutants of presenilin-1 protein and presenilin-2 protein, which cause the familial Alzheimer's disease, possibly trigger the onset of Alzheimer's disease by the increase of Aβ 42 which is considered to play a significant role in the formation of senile plaque. A trans-genic mouse transferred with a gene encoding the mutant presenilin-1 protein was created (Duff K. et al.: Nature, Vol. 383, P710, 1996, Borchelt DR. et al.: Neuron, Vol. 17, P1005, 1996 and Citron M. et al.: Nature Med., Vol. 3, P67, 1997). It was reported that Aβ 42 in the brain of the trans-genic mouse selectively increased. These results are strong supports of the possibility that mutants of presenilin-1 protein and presenilin-2 protein causing the familial Alzheimer's disease increase Aβ 42 which possibly has significant roles in the formation of senile plaque, thereby develop Alzheimer's disease. However, no description is given about histological study of the mouse's brain in the above reports on the trans-genic mouse; which presumably due to no observation of remarkable histological change in the brain of the trans-genic mouse.

**[0014]** Generally, trans-genic animals are useful as a means of analyzing functions of a target gene *in vivo*. However, it is technically difficult to control the expression of a transferred gene quantitatively, tissue specifically, or time specifically during development. There is also a problem in that two different gene products are present as a mixture in the trans-genic animals since a gene inherently possessed by the animal still works for normal expression, and functions of a transferred gene cannot be sufficiently analyzed. Furthermore, when the transferred gene is subjected to particularly excessive expression, functions not inherently performed in vivo may appear in trans-genic animals, which results in a defect of possible confusion in analysis of constructed gene-mutated animals.

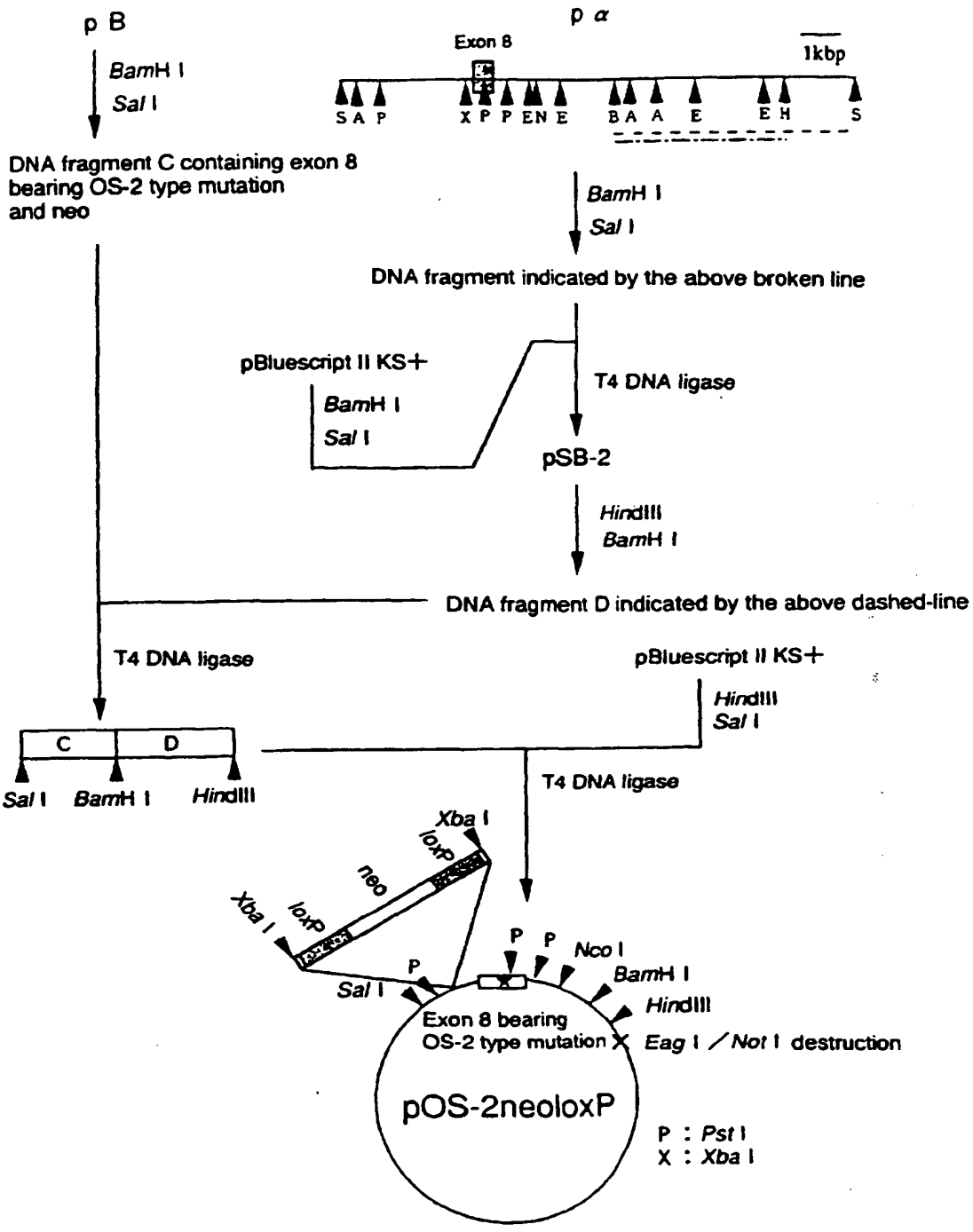
**[0015]** Apart from trans-genic animals, knockout animals may also be used as a means of analyzing functions of a target gene. In a knockout animal, a target gene inherently possessed by the animal is artificially destroyed so as to be dysfunctional. A detailed analysis of knockout animals may reveal functions of a target gene in vivo. However, particular changes in knockout animals created as homozygote sometimes fails to appear, since the functions of the other gene products in the knockout animal may substitute for that of the destroyed gene products. Furthermore, there is also a problem in that an animal as homozygote may sometimes be lethal because the destroyed gene product is essential to the animal's development and growth, whilst thorough analysis of gene functions of an animal as viable heterozygote is practically impossible.

## Disclosure of the Invention

**[0016]** An object of the present invention is to provide, for creation of an animal pathologic model of Alzheimer's disease, an animal as a pathological model whose pathologic conditions is closer to those of a patient with Alzheimer's disease, instead of a trans-genic animal having the aforementioned defects. More specifically, the object of the present invention is to provide a gene-mutated animal capable of expressing a mutant presenilin protein in the brain by transfer of a mutant of a presenilin gene which is believed to be a causal gene of Alzheimer's disease (a mutant presenilin gene) according to a homologous recombination technique. Further objects of the present invention are to provide a method of producing said gene-mutated animal; a plasmid useful for the aforementioned production method; and a method for evaluating a substance or an agent effective for preventive and/or therapeutic treatment of Alzheimer's disease using the aforementioned gene-mutated animal.

**[0017]** In order to reveal roles of presenilin-1 protein and mechanism of the onset of Alzheimer's disease by the mutation of presenilin-1 gene, the inventors of the present invention created a knockin mouse in which presenilin-1

Fig. 7



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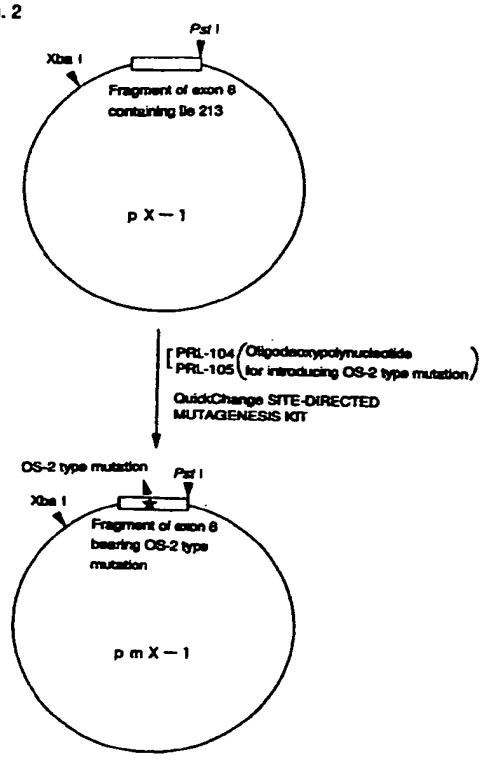
# (54) GENE MUTANT ANIMALS

(57) A gene-mutated animal such as a mouse which comprises a mutant prsenilin-1 gene comprising a DNA having a sequence encoding a mutant presenilin-1 protein in which an amino acid is substituted with a different amino acid in an amino acid sequence of a presenilin-1 protein; for example, a mutant presenilin protein in which isoleucine at position 213 is substituted with an amino acid other than isoleucine, e.g., threonine, in a mouse presenilin-1 protein. The animal is useful as an animal model which has pathological conditions closer to a human patient with Alzheimer's disease.

Fig. 2




EP 1 044 605 A1

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP99/00015

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl⁶ A01K67/027, A61K45/00, C12N15/12, G01N33/15

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl⁶ A01K67/027, A61K45/00, C12N15/12, G01N33/15

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
BIOSIS PREVIEWS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X<br>Y | K. Duff et al., Nature, vol. 383, p.710-713 (1996) | 1-4, 12-15, 17<br>18, 33, 36-45<br>5-11, 16,<br>19-32, 34, 35 |
| X<br>Y | D.R. Borchelt et al., Neuron, vol. 17, p.1005-1013 (1996) | 1-4, 12-15, 17<br>18, 33, 36-45<br>5-11, 16,<br>19-32, 34, 35 |
| X<br>Y | M. Citron et al., Nature Medicine, vol. 3(1), p.67-72 (1997) | 1-4, 12-15, 17<br>18, 33, 36-50<br>5-11, 16,<br>19-32, 34, 35 |
| Y | J. Hardy et al., TINS, vol. 20(4), p.154-159 (1997) | 1-50 |
| Y | K. Kamino et al., Neuroscience Letters, vol.208, p.195-198 (1996) | 1-50 |

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 25 March, 1999 (25. 03. 99) | 6 April, 1999 (06. 04. 99) |
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国際調査報告

国際出願番号　PCT／JP99／00015

A.　発明の属する分野の分類（国際特許分類（IPC））

Int. Cl.⁶ A01K67／027, A61K45／00, C12N15／12, G01N33／15

B.　調査を行った分野

調査を行った最小限資料（国際特許分類（IPC））

Int. Cl.⁶ A01K67／027, A61K45／00, C12N15／12, G01N33／15

最小限資料以外の資料で調査を行った分野に含まれるもの

国際調査で使用した電子データベース（データベースの名称、調査に使用した用語）

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| X | K. Duff et al., Nature, vol. 383, p. 710-713 (1996) | 1-4, 12-15, 17 |
| Y | | 18, 33, 36-45 |
| | | 5-11, 16, |
| | | 19-32, 34, 35 |
| X | D. R. Borchelt et al., Neuron, vol. 17, p. 1005-1013 (1996) | 1-4, 12-15, 17 |
| Y | | 18, 33, 36-45 |
| | | 5-11, 16, |
| | | 19-32, 34, 35 |

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